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Validation of a Real-Time Reverse Transcriptase–PCR Assay for the Detection of H7 Avian Influenza Virus

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SUMMARY. This report describes the validation of an avian influenza virus (AIV) H7 subtype-specific real-time reverse transcriptase–PCR (rRT-PCR) assay developed at the Southeast Poultry Research Laboratory (SEPRL) for the detection of H7 AI in North and South American wild aquatic birds and poultry. The validation was a collaborative effort by the SEPRL and the National Veterinary Services Laboratories. The 2008 H7 rRT-PCR assay detects 10^1 50% embryo infectious doses per reaction, or 10^3–10^4 copies of transcribed H7 RNA. Diagnostic sensitivity and specificity were estimated to be 97.5% and 82.4%, respectively; the assay was shown to be specific for H7 AI when tested with >270 wild birds and poultry viruses. Following validation, the 2008 H7 rRT-PCR procedure was adopted as an official U.S. Department of Agriculture procedure for the detection of H7 AIV. The 2008 H7 assay replaced the previously used (2002) assay, which does not detect H7 viruses currently circulating in wild birds in North and South America.

RESUMEN. Validación de un método de transcripción reversa y reacción en cadena de la polimerasa en tiempo real para la detección del virus de la influenza aviar H7.

Este informe describe la validación de un método de transcripción reversa y reacción en cadena de la polimerasa en tiempo real específico para el subtipo H7 del virus de la influenza aviar (RRT-PCR) desarrollado en el Laboratorio de Investigación Avícola del Sureste (con las siglas en inglés SEPRL) para la detección de influenza aviar H7 en aves silvestres acuáticas y aves de corral de Norteamérica y de América del Sur. La validación fue un esfuerzo de colaboración entre el SEPRL y el Servicio Nacional de Laboratorios Veterinarios. El método rRT-PCR para H7 del año 2008 detecta 10^1 dosis infecciosas 50% por reacción, o 10^3–10^4 copias de ARN transcrito del gene H7. La sensibilidad y la especificidad diagnóstica se estimaron en 97.5% y 82.4%, respectivamente, el ensayo ha demostrado ser específico para el virus de la influenza aviar H7 cuando se probó con más de 270 virus de aves silvestres y aves de corral. Tras la validación, el método rRT-PCR para H7 del año 2008 fue adoptado como procedimiento oficial del Departamento de Agricultura de los Estados Unidos para la detección del virus de la influenza aviar H7. El ensayo del 2008 sustituyó al procedimiento para detectar al subtipo H7 utilizado anteriormente (2002), que no detecta los virus H7que circulan actualmente en aves silvestres en Norteamérica y en América del Sur.

Key words: avian influenza, poultry, real-time RT-PCR, surveillance, wild birds

Abbreviations: AI = avian influenza; AICAP = Prevention and Control of Avian Influenza in the United States; CL = cloacal; DPI = days postinoculation; DxSN = diagnostic sensitivity; DxSP = diagnostic specificity; EID_{50} = 50% embryo infectious dose; HPAI = highly pathogenic avian influenza; LPAI = low pathogenicity avian influenza; NVSL = National Veterinary Services Laboratories; OP = oropharyngeal; rRT-PCR = real-time reverse transcription–PCR; SPF = specific pathogen free; TN = true negative; TP = true positive; USDA = U.S. Department of Agriculture; VI = virus isolation

As part of the 2006 U.S. Department of Agriculture (USDA) and Department of the Interior interagency surveillance for H5N1 highly pathogenic avian influenza (HPAI) and the Prevention and Control of Avian Influenza in the United States (AICAP) surveillance programs, more than 164,000 swab specimens were screened by a matrix-based real-time reverse transcription–PCR (rRT-PCR) assay for detection of avian influenza (AI) virus in all 50 states. Specimens positive for AI by the matrix gene assay were further tested for H5 and H7 by subtype-specific rRT-PCR assays. The isolation of H7 AI virus from H7 rRT-PCR negative swabs and a 0% H7 rRT-PCR detection rate indicated that the 2002 USDA H7 rRT-PCR assay did not detect the lineage of H7 currently circulating in wild birds. The 2002 H7 assay developed by Spackman *et al.* (8) had been used by the USDA for detection of H7 AI in live bird market poultry as well as in commercial poultry in Virginia (2002), Delaware, and Connecticut (2004), but it failed to detect H7N9 in South Dakota (2008), HPAI H7N3 in Chile (2002), and low pathogenicity AI (LPAI) in wild aquatic birds (2006). A new (2008) assay for the detection of current North and South American lineages of H7 was validated to determine assay performance characteristics, including analytical and diagnostic sensitivity and specificity, limit of detection, and performance with alternative chemistry and real-time PCR platforms.

MATERIALS AND METHODS

Viruses. The avian and equine influenza virus strains used in the study as well as the avian respiratory pathogens obtained from the National Veterinary Services Laboratories (NVSL) reference collection were propagated in embryonated specific-pathogen-free (SPF) chicken eggs and subtyped with conventional hemagglutination inhibition and neuraminidase inhibition procedures (9). With the exception of A/Chile/176822-1/02 (H7N3) and A/British Columbia/CN-7-3/04

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Table 1. Limit of detection in terms of 50% embryo infectious dose (EID_{50}) for H7N1 ($10^{8.1}$ EID_{50}/ml) and H7N2 ($10^{7.4.5}$ EID_{50}/ml) with AgPath-ID™ and Qiagen One-Step RT-PCR® chemistries and AB7500Fast, AB7900HT, Cepheid SmartCycler®, and Roche LightCycler480™ real-time instruments. Testing was conducted in replicate.

	Limit of detection (in terms of EID_{50} per reaction for H7N1 and H7N2 avian influenza) with AgPath and Qiagen chemistries			
Instrument	H7N1 AgPath	H7N2 AgPath	H7N1 Qiagen	H7N2 Qiagen
AB7500Fast	10^{0-1} EID_{50}	10^{-1} EID_{50}	10^{1-2} EID_{50}	10^{0-1} EID_{50}
AB7900HT	10^{0} EID_{50}	10^{0} EID_{50}	10^{1} EID_{50}	10^{0} EID_{50}
Cepheid SC	10^{1} EID_{50}	10^{0} EID_{50}	10^{0} EID_{50}	10^{-1} EID_{50}
Roche LC480®	10^{-10} EID_{50}	10^{-10} EID_{50}	10^{0-1} EID_{50}	10^{-10} EID_{50}

(H7N3), all viruses were LPAI; the Chile and British Columbia H7N3 isolates were HPAI. In addition, 82 H7 AIVs (H7N1, H7N3, H7N4, H7N7, and H7N8 subtypes) and 157 non–H7 AI viruses isolated from wild birds were tested to evaluate analytical specificity. The H and N subtypes for all 239 wild bird isolates from the USDA/Department of the Interior and AICAP surveillance programs were determined by conventional hemagglutination inhibition/neuraminidase inhibition and/or molecular subtyping procedures. Tenfold serial dilutions of titered H7 reference viruses A/TY/Ont/18-2/00 (H7N1: $10^{8.1}$ 50% embryo infectious doses [EID_{50}]/ml) and A/CK/NY/273874/03 (H7N2: $10^{7.45}$ EID_{50}/ml) were tested in triplicate to determine the EID_{50} per reaction.

Diagnostic specimens and RNA extraction. Clinical specimens consisted of cloacal (CL) and oropharyngeal (OP) swabs collected from 4-to-5-wk-old SPF chickens experimentally inoculated with 10^{6} EID_{50} A/TY/ONT/18-2/00(H7N1) as well as diagnostic poultry CL and OP swabs ($n = 1578$). From experimentally inoculated chickens, CL and OP swabs specimens were collected twice daily (morning and evening) between 1 and 8 days postinoculation (DPI) in 3.5 ml of brain heart infusion broth. Swabs were swirled in brain heart infusion broth to disperse contents, squeezed against the side of the tube to eliminate liquid, and then discarded. Swab supernatants were held at 4 C for up to 24 hr and were then processed for RNA extraction. Nucleic acid was extracted from 50 μl of clarified swab supernatant with MagMAX™ AI/ND Viral RNA Isolation Kit (Ambion, Austin, TX). Nucleic acid extraction was conducted according to procedures described in the USDA/NVSL protocol "Real-Time RT-PCR for the Detection of Avian Influenza Virus and Identification of H5 and H7 subtypes in Clinical Samples" (AVPRO1510.03, available upon request) with a KingFisher (Thermo, Ontario, Canada) 96-well magnetic particle processor. The magnetic particle processor was programmed according to USDA/NVSL standard operating procedure AVSOP1522.01 (available upon request).

Virus isolation. Virus isolation was conducted in 9-to-11-day-old SPF chicken embryos with conventional procedures, as described previously (9). Briefly, 2.0 ml of swab supernatant was diluted in antibiotic suspension, incubated for 1 hr at room temperature, and inoculated (0.3 ml per embryo) into the allantoic sac of 9-to-11-day-old embryonating eggs (9). A second blind passage was made for all specimens that were negative for virus upon completion of the first passage and positive by rRT-PCR.

rRT-PCR. Nucleotide sequence for the primers and probe for the 2008 H7 assay were described by Spackman *et al.* (7). Validation testing conducted at the NVSL used 2.5 pmol/reaction of each primer and 1.5 pmol/reaction of FAM/black hole quencher probe. A 25-μl reaction volume, as described in USDA/NVSL AI rRT-PCR protocol (AVPRO1510.03, available upon request), was used for both the AgPath-ID™ (Ambion) and Qiagen One-Step RT-PCR® (Qiagen, Valencia, CA) kits with Applied Biosystems (AB)7500Fast (Applied Biosystems, Foster City, CA), AB7900HT, SmartCycler® (Cepheid, Sunnyvale, CA), and LightCycler480™ (Roche, Mannheim, Germany) real-time PCR instruments. Multiple PCR platforms and chemistries were compared with the serially diluted H7N1 and H7N2 reference viruses as part of the analytical sensitivity portion of the study. All diagnostic and analytical specificity validation testing was conducted with AgPath-ID™ chemistry and AB7500 instrumentation.

Phylogenetic analysis. Sequences of H7-subtype viruses isolated from wild birds in 2007 were generated at the NVSL as part of the bench validation for the 2008 H7 assay. The remaining sequences were obtained from the GenBank. An H7 phylogenetic tree was constructed using nucleotide sequences from the hemagglutinin gene and Lasergene software, version 8.0 (DNA Star, Madison, WI). Phylogenetic inference was conducted using the neighbor-joining method of inference with 1000 bootstraps.

RESULTS

The analytical sensitivity for the 2008 H7 rRT-PCR assay has been reported by Spackman *et al.* (7) to be 10^{1} EID_{50} per reaction, or 10^{3}–10^{4} copies of transcribed RNA. Limit of detection studies conducted with AgPath-ID™ and Qiagen One-Step RT-PCR® chemistries with AB7500Fast, AB7900HT, Roche LightCycler480®, and Cepheid SmartCycler® instrumentation supported the previously reported analytical sensitivity data (Table 1). The 2008 H7 assay is comparable to the USDA/NVSL matrix gene rRT-PCR assay. The AB7500Fast, AB7900HT, and Roche LightCycler480® platforms are approximately 10 times more sensitive than the Cepheid SmartCycler® instrument with AgPath-ID™ One-Step RT-PCR chemistry, and the Cepheid SmartCycler® instrument is approximately 10 times more sensitive than the alternative platforms with Qiagen One-Step RT-PCR® chemistry.

The 2008 H7 assay was found to be specific for North American and South American lineages of H7 AI and did not amplify AI of the H1–H6 and H8–H15 subtypes. The positive/negative detection limits for reference H1–15 AI viruses and poultry respiratory pathogens are shown in Table 2. One nonpoultry H7 reference virus, Eq/Prague/56 (H7N7), tested negative for H7 viral RNA by the 2008 H7 assay. In addition to the viruses listed in Table 2, 239 AI viruses isolated from North American wild birds were tested by the 2008 H7 assay. Of the 239 AI viruses tested, 100% (82 of 82) of the H7 subtype were detected by the 2008 H7 rRT-PCR assay: the remaining 157 non-H7 viruses were negative.

The diagnostic sensitivity (DxSN) and diagnostic specificity (DxSP) were determined by associating the 2008 H7 positive/negative categoric data with the known infection status of each animal using a two-way (2 × 2) table (Table 3). The known infection status of each animal was determined by virus isolation (VI), the gold standard for detection of AI virus. The true positive (TP) and true negative (TN) specimens were those specimens that tested positive for H7 AI by VI and the 2008 H7 test and those that tested negative for both tests, respectively. Specimens classified as false positive and false negative were those that were in disagreement with VI. Diagnostic sensitivity was calculated as TP/(TP + false negative), and DxSP was calculated as TN/(TN + false positive). The DxSN and DxSP of the 2008 H7 rRT-PCR assay as compared to VI

Table 2. Panel of reference H1–H15 avian influenza viruses and avian respiratory pathogens. Isolate name, subtype, and real-time RT-PCR positive/negative result for the 2008 H7 assay are listed.

Isolate	Subtype	+/−
DK/MN/764-1489/81	H1N2	−
A/NJ/8/76-Eq1	H1N7	−
Mallard/ALB/77/77	H2N3	−
Waterfowl/GA/03	H2N9	−
DK/MN/LQWR604/79	H3N4	−
Mallard/ALB/311/85	H3N6	−
DK/Czech/56	H4N6	−
DK/England/62	H4N8	−
MSwan/MI/451072-2/06	H5N1	−
DK/PA/454069/06	H5N1	−
CK/WA/13413/84	H5N2	−
CK/CA/101247/01	H6N2	−
Emu/TX/4259/93	H7N1	+
CK/NY/273874/03	H7N2	+
TY/UT/24721-10/95	H7N3	+
TY/OR/71	H7N3	+
CK/Chile/176822-1/02	H7N3	+
CK/BC/314514/04	H7N3	+
EQ/Prague/56	H7N7	−
Mallard/AK/495762-38/06	H7N8	+
TY/NE/505577/07	H7N9	+
TY/Ontario/6118/67	H8N4	−
TY/CA/6889/80	H9N2	−
CK/Germany/N/48	H10N7	−
DK/England/56-Bel	H11N1	−
Mallard/LALB/83	H12N5	−
Gull/MD/704/77	H13N6	−
Mallard/Gurjev/263/82	H14N5	−
Shearwater/Aust/1/73	H15N9	−
Avian paramyxovirus	APMV 1–4, 6–9	−
Infectious bronchitis	Mass	−
Avian metapneumovirus	Subtype C	−
Infectious laryngotrachitis virus		−

were estimated to be 97.5% and 82.4%, respectively. The positive/negative virus isolation and rRT-PCR data for the experimentally inoculated chickens included in the estimation of DxSN and DxSP were from 1–8 DPI CL and OP swabs. In the early phase of collection (2–3 DPI) many of the swabs, especially the OP swabs, were positive by H7 rRT-PCR and negative by VI. During the later phase of collection (7–8 DPI), a significant percentage of CL swab specimens were positive by H7 rRT-PCR and negative by VI. The greatest degree of correlation between the rRT-PCR and VI test occurred between 4 and 6 DPI.

The 2008 H7 assay was shown to detect North and South American lineages of H7 AI isolated from poultry and wild aquatic birds. The 2002 H7 assay was used for the detection of H7 AI from 2002 to 2007 in live bird markets in New York, New Jersey, and other northeastern states as well as for the detection of infections in commercial poultry in Delaware, Connecticut, and Virginia. The 2002 assay failed to detect a HP H7N3 in commercial poultry in Chile in 2002 and in British Columbia in 2004 as well as a LPAI H7N9 in commercial turkeys in South Dakota in 2007 (Fig. 1). In addition, the 2002 North American H7 assay failed to detect H7-subtype influenza from any of the AI matrix-positive specimens collected during the 2006 wild bird and AICAP surveillance periods. The 2008 H7 assay has increased the specificity of the USDA/NVSL procedure to include wild bird and poultry lineages circulating in North and South America (Fig. 1).

Table 3. Number of diagnostic swab specimens testing positive/negative for H7 AI by the 2008 H7 rRT-PCR assay and VI. Specimens included oropharyngeal and cloacal swabs collected from experimentally inoculated chickens and diagnostic poultry swab specimens (n = 1578).

	VI Positive	VI Negative		
rRT-PCR Positive	316 TP	221 FP	537	DxSN = 97.5%
rRT-PCR Negative	8 FN	1033 TN	1041	DxSP = 82.4%
Totals	324	1254		

DISCUSSION

rRT-PCR is shown to be an effective tool for the surveillance of AI in both commercial poultry and wild aquatic birds throughout the world (1,2,3,4,5,6). Surveillance and control programs normally use a matrix gene rRT-PCR assay for the detection of type A influenza and H5 and H7 subtype-specific assays for the detection H5 and H7 AI, which is critical for control of the disease and unhindered trade. As an effective surveillance tool, rRT-PCR tests require analytical and diagnostic validation as well as continued evaluation to assure that primers and probes will detect current lineages of AI. An essential component of continued evaluation of the assay is the isolation and subtype identification of AI viruses from all matrix-positive specimens. The 2008 H7 assay detects the current lineages of H7 circulating in North and South America, but it does not detect Eurasian lineages of H7 AI (7) or the H7 lineage of equine influenza. The 2008 H7 assay has been shown to be specific for H7 and does not detect other subtypes of AI or other poultry respiratory pathogens. In 2008 the NVSL received 281 presumptive H7 specimens for confirmation testing; 142 of the 281 presumptive H7 specimens were confirmed to be positive by the 2008 H7 assay.

A critical component of USDA preparation for an outbreak of HPAI is to increase the testing capacity of the National Animal Health Laboratory Network. Evaluation of alternative 96-well real-time platforms will increase testing capacity by allowing National Animal Health Laboratory Network labs to use alternative platforms that have been shown to be equivalent. The AB7500Fast, AB7900HT, Roche LightCycler480®, and Cepheid SmartCycler® instruments are similar in sensitivity, detecting approximately 10^{0-1} EID_{50} with AgPath-ID™ and Qiagen One-Step RT-PCR® chemistries. Robustness of the 2008 H7 assay was evaluated with an intra-assay and interassay variability study retrospective to analytical and diagnostic validation. Five within-run replicates demonstrated a $<$1.0 cycle threshold (Ct) difference, with tenfold serial dilutions of a reference virus over the linear range of the assay. Interassay variability studies demonstrated good between-run repeatability with the four previously identified platforms and two chemistries over the linear range of the assay.

The DxSN and DxSP of the assay were 97.5% and 82.4%, respectively, when compared to VI. The lower DxSP value is likely influenced by the inclusion of swabs from experimentally inoculated chickens in the validation study, in which viral RNA can be detected by RT-PCR but virus cannot be isolated, an insufficient number of negative specimens, and the use of a gold standard with a less

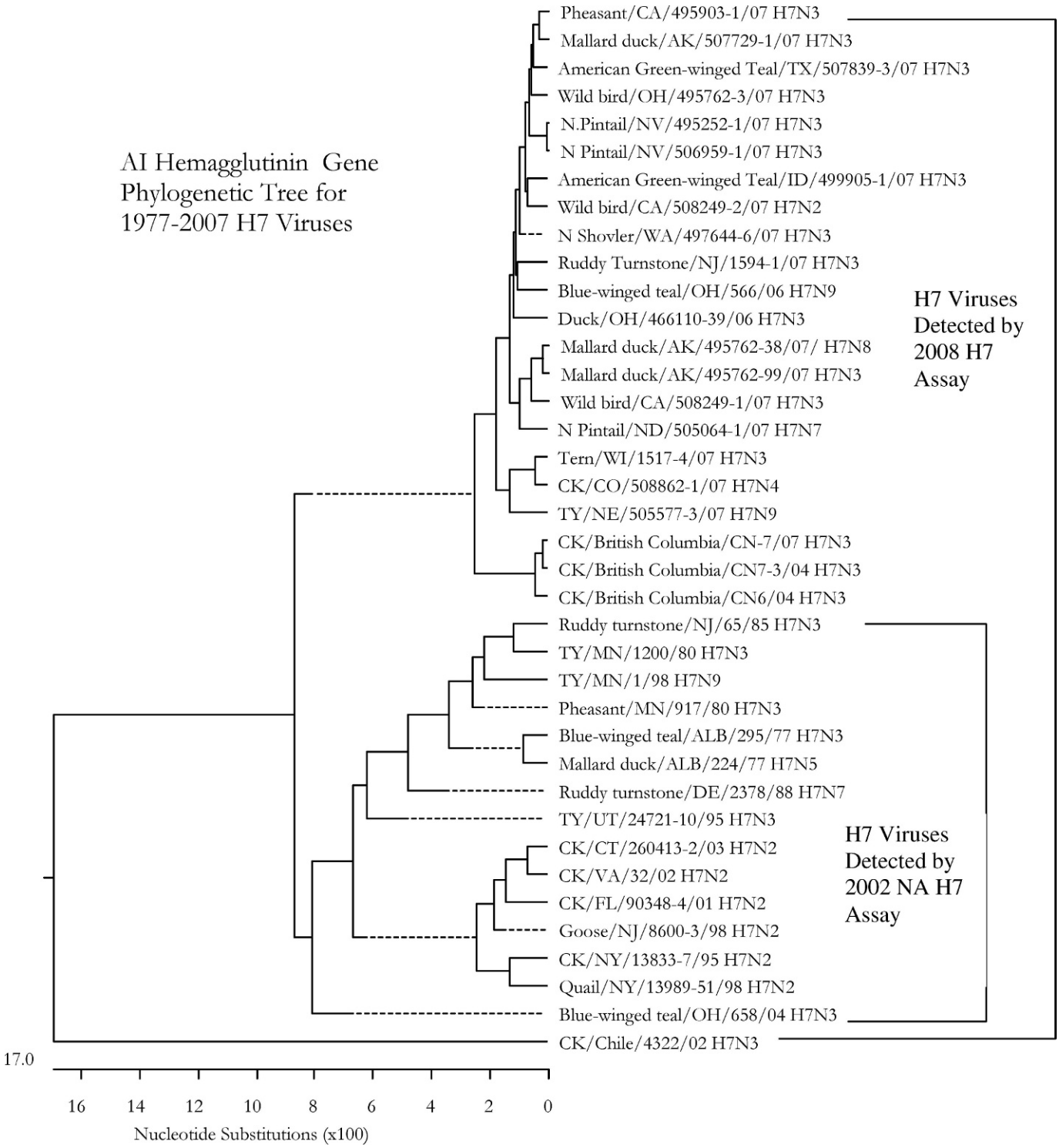

Fig. 1. Phylogenetic tree for the hemagglutinin gene of H7 viruses isolated between 1997 and 2007.

sensitive indicator system, the hemagglutination test. Conventional VI procedures use the hemagglutination test for identification of a hemagglutinating virus. The hemagglutination test detects approximately 4 logs of virus, as compared to the 10^1 EID_{50} detected by rRT-PCR. The 2008 H7 assay has replaced the 2002 H7 assay for the detection of H7 in commercial poultry and wild aquatic birds; as a critical component of the USDA AI surveillance program, the assay performance characteristics will continue to be evaluated.

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